INVESTIGATION OF GENES ASSOCIATED WITH THE WHITE COAT COLOR IN TIGERS

An Honors Fellow Thesis

by

EMILEE ANN LARKIN

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

May 2012

Major: Biomedical Sciences

INVESTIGATION OF GENES ASSOCIATED WITH THE WHITE COAT COLOR IN TIGERS

An Honors Fellow Thesis

by

EMILEE ANN LARKIN

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

Approved by:

Research Advisor: Jan Janecka
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

May 2012

Major: Biomedical Sciences

ABSTRACT

Investigation of Genes Associated with the White Coat Color in Tigers. (May 2012)

Emilee Ann Larkin
Department of Veterinary Medicine and Biomedical Sciences
Texas A&M University

Research Advisor: Dr. Jan Janecka
Department of Veterinary Medicine and Biomedical Sciences

The white phenotype in tigers is highly sought after by zoos and private sanctuaries. There are enough animals in the captive population to avoid severe inbreeding; however close relatives are often bred together to produce the elusive white phenotype. Heritable disorders in white tigers have been noted, for example strabismus, cleft palate, and spinal and facial defects. These are linked to the white phenotype and likely caused by inbreeding. It is important to determine the genetic basis of the white coat color and associated disorders so that breeding could be modified to avoid these detrimental phenotypes.

We used information obtained from other species regarding the genes found to be involved in similar coat color patterns to select two likely candidates for the white phenotype in tigers, melanocortin-1-receptor (MC1R) and Agouti Signaling Protein (ASIP). Both MC1R and ASIP affect the relative amounts of pigments produced by the melanocytes. MC1R controls the peptide hormones melanocortins that determine the

type of melanin produced by the melanocyte. ASIP is the antagonist for MC1R, deactivating alpha-melanoctye stimulating hormone, which leads to increased pheomelanin production, while loss of activity of ASIP results in eumelanin.

We isolated DNA from four white, one snow white, and fourteen orange tigers. We then Sanger sequenced ASIP exon 1 and 2 for these various tigers as well as several other felid species. Sequences were aligned in MEGA and the nucleotide and amino acid differences were compared. These ASIP sequences were then compared to other felids and with several families of primates. This analysis showed no difference in the two ASIP exons between the tiger coat colors. This leads us to believe that the coding sequence of exon 1 and 2 of ASIP is not responsible for the white coat color in tigers.

ACKNOWLEDGMENTS

I would like to thank everyone in the Texas A&M Molecular Cytogenetics and Genomics Laboratory for helping and supporting me in my time with them. I could not have asked for a better group of people with whom to start my journey into science with. I would especially like to thank Dr. Bhanu Chowdhary, Dr. Samantha Steelman and Dr. Jan Janecka for pushing me to be the best I can be and always being there for questions. I would also like to thank my family for putting up with random discussions on genetics where I went on and on while they had no idea what I was talking about.

I would also like to thank everyone who kindly provided samples for this project including the San Francisco Zoo, In-sync Exotics (Vicky Keahey), Big Cat C.A.R.E, (Heidi Riggs Berry), Sierra Endangered Cat Haven (Dale Anderson), Big Cat Rescue, Tiger Creek, the Exotic Feline Rescue Center, Ferdinand and Antonin Fercos, and REXANO (Zuzana Kukol). Without you studies like this would not be possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Few things are as captivating as watching a tiger slink through the jungle, stalking its prey. Animals above us on the food chain have long entranced humans with their prowess and strength. Yet the tiger, *Panthera tigris*, is disappearing from its natural habitat at an alarming and ever increasing rate (Dinerstein et al, 2007). The loss of tiger populations in the wild makes maintaining the integrity of our captive populations even more important.

Captive populations often lead to reduction in the gene pool. This is due to there being so few individuals to choose from for mating that inbreeding is inevitable. This can lead to detrimental mutations being fixed in the population as a whole. This is especially prevalent in the white tiger population. Essentially this is caused by a population bottleneck. Population bottlenecks occur when a large number of individuals in a population suddenly die or become sterile, or as with white tigers a small number of animals with the desired trait were captured to use in breeding programs. The few individuals left are the parents of all the future progeny. This means that if these individuals are heterozygotes, the likelihood of a homozygous recessive offspring is increased. This can lead to offspring with severe defects and result in these defects

---

This thesis follows the style of Molecular Phylogenetics and Evolution.

becoming common in the population through genetic drift.

The first white tiger was seen in the wild in India in 1916; however, it was not until 1951 that a white tiger was captured and placed in captivity (Thornton et al. 1966). This tiger was Mohan, the progenitor of the white tigers alive today. Mohan was found in the region of India called Rewa (now Madhya Pradesh) by the Maharaja. Mohan was placed with a wild-bred orange tigress, named Begum, and they produced ten cubs over three litters (Thornton et al. 1966). All of these cubs were orange. Begum was also bred four times with one of these original offspring; these unions produced twelve cubs all of which were orange. Mohan was then bred to one of his daughters four times. This union was also productive and resulted in a total of eleven white and three orange cubs. Mohan was again bred to one of these cubs (from the F2 generation) and that union produced six white cubs and no orange cubs. This pedigree shows that the white mutation is most likely recessively inherited and follows a classical Mendelian genetic distribution from a single autosomal locus (Thornton et al. 1966).

The white phenotype is highly desired by zoos and breeders. Indeed, most of the offspring of Mohan and Begum ended up in zoos all across the world. Unfortunately, the best way to guarantee getting a white tiger is to breed two individuals containing that allele together, which often means breeding direct relatives with each other. Inbreeding depression could be the reason why the white phenotype is often associated with several serious morphological defects. Varying disorders in white tigers have been noted, for

example strabismus (crossed eyes), cleft palate, as well as spinal and facial defects (Roychoudhury and Sankhala 1979). However with proper breeding strategies and the ability to determine which individuals carry the alleles that cause the morphological disorders, we could eventually breed these defects out of the white phenotype and thus keep these beautiful and majestic animals around for our children's children.

The white coat color is believed to be the result of repression of one of the melanin because of a recessive autosomal mutation. Melanins, pigments that give color to hair, skin and feathers, exist in two different types. The first is called "eumelanin" and is responsible for black and brown colors. The second is "pheomelanin," which is responsible for red and yellow (Hill et al. 1997). The precursor for the melanocyte, cells that actually produce the pigments, is a melanoblast. These migrate from the neural crest, a collection of cells formed during embroyogenesis, to the hair or feathers. There they mature into melanocytes and begin producing pigments (Mills et al. 2009). Melanocytes are able to switch between eumelanin and pheomelanin production, meaning that the same one can produce both types.

Candidate genes for coat color

While little research has been done on the genetics basis for the white coat color in tigers; a significant amount has been explored comparable phenotypes in other mammals. In rabbits, *Oryctolagus cuniculus,* mice, *Mus musculus*, and rats, *Rattus noregicus*, the equivalent phenotype is called "chinchilla," while in domestic cats, *Felis catus*, it is

called “silver” (Robinson 1968). Individuals with these phenotypes are characterized by a repression in pheomelanin, leading to a vast reduction in color, while eumelanin is either unaffected or is only slightly dulled. These specially colored mammals are directly comparable to the white coat color in tigers. The white coat color is often mistakenly named an albino. However, this is incorrect (Robinson 1968). Albinos are characterized by a total reduction of pigments, whereas the white tiger still has eumelanin. In this sense they can be looked upon as halfway between a normally colored animal and an albino. Albino tigers have been seen in the wild, going as far back as 1922 (Thornton et al. 1966). These tigers were not only completely white, but also exhibited the classical red eyes associated with albinism. Based on these comparable phenotypes, we identified two genes that stand as good candidates for being linked to the white coat color in tigers and we examined their sequence in both white and orange tigers to determine if there was a common mutation. These two genes are Melanocortin-1-receptor gene (MC1R) and Agouti Signaling Protein gene (ASIP). The interaction between these two proteins is responsible for switching between eumelanin and pheomelanin production in the melanocytes (Mills et al. 2009).

Melanocortin-1-receptor gene

MC1R has control over melanocortins, which are peptide hormones that control pigment production (Rieder et al, 2001). MC1R is what actually controls the manufacture and production of the pigments. Because MC1R has such a large effect on pigment production many studies have looked at MC1R in a variety of different animals.

In rabbits a study examined the chinchilla color and reported it to be linked to the melanocortin 1 receptor gene (MC1R) at the *extension* locus (Fontanesi et al. 2006). Evidence also links the MC1R gene with melanism in jaguars (Eizirik et al 2003). Melanism is a pigmentation pattern where the skin, hair or feathers of an animal are completely or almost completely black, due to an overproduction of eumelanin. It has also been linked to the amber color in the Norwegian Forest Cat (Peterschmitt et al. 2009). These extremely different phenotypes result of two identified mutations. The first is called the E^D mutation. This is dominantly inherited and produces constitutively active receptors, which is what causes melanism. However, the second type, called the *e* mutation, is recessively inherited and leads to a variety of coat colors, such as amber (Peterschmitt et al. 2009). We chose this gene because it has been proven to affect pheomelanin and eumelanin production and because the phenotypes appear to be highly dependent on the specific type of mutation. MC1R is an intronless gene with a coding sequence of 954 base pairs (Eizirik et al. 2003).

Agouti signaling protein

ASIP is the peptide antagonist for MC1R (Rieder et al, 2001). It works by deactivating alpha-melanocyte stimulating hormone and allows the melanocyte to create pheomelanin. When ASIP is not functioning properly, alpha-melanocyte stimulating hormone remains activated and only eumelanin is produced (Rieder et al. 2001, Mills et al. 2009). ASIP has been implicated as leading to melaninsm in horses, *Equus caballus*, white and black coat colors in sheep, *Ovis aries*; black and tan coats in rabbits; the yellow color in mice; the

black coat color in German Shepherd dogs, *Canis familiaris*; and the black color in the domestic cat (Rieder et al. 2001; Norris et al. 2012; Fontanesi et al. 2010, Bultman et al. 1992; Kerns et al. 2004; Eizirik et al. 2003). ASIP is one of the most recognized genes that affect pigment color and production and we therefore decided to begin our analysis with it.

This research will provide insight into several interesting aspects of tiger biology and conservation. By potentially identifying the mutation that leads to the white phenotype we will gain a better understanding of inheritance patterns, and the genetic basis and evolution of color-related genes in felids. This understanding will also provide insight into another piece of the pheomelanin pathway. Understanding this pathway will lead to a better comprehension of color development in skin and hair. In addition, melanin synthesis, or the lack thereof, is currently believed to play a major role in the development of certain skin cancers (Lin et al. 2007). Therefore, any increase in our knowledge in this area could be very important towards the better understanding of cancer.

Lastly this research will be the first step to identifying the genes linked with defects seen in the white tigers. By identifying these genes we will be able to better advise breeders on which animals to breed together to achieve the most genetically sound individuals. This would result in the captive populations of tigers staying as genetically viable as possible and assist in keeping the captive populations true to their wild counterparts.

Therefore we investigated variation in the ASIP gene and its relationship to the white coat color in tigers. We also performed a phylogenic study of ASIP comparing the sequences of exon 1 and 2 among multiple felids and primates.

CHAPTER II

METHODS

Samples

Blood was obtained from four white, one snow white, fifteen orange tigers, one liger (*Panthera leo* X *Panthera tigris)*, two snow leopards (*Panthera uncia*), two leopards (*Panthera pardus*), two cougars (*Puma concolor*), one lion (*Panthera leo*), and one jaguar (*Panthera onca*). These samples were kindly provided from a number of different breeders, zoos, and private owners, such as Big Cat C.A.R.E., In-sync Exotics, Big Cat Rescue, Tiger Creek and the Exotic Feline Rescue Center, and REXANO. DNA was isolated following the standard PureGene DNA isolation protocol. Each sample was collected in accordance with the College of Veterinary Medicine Clinical Research Review Committee (CRRC permit #10-44 J. Janecka).

PCR methodology

Primers were designed using Primer3 and the BLAT cat genome (Rozen and Skaletsky 2000; Fujita et al. 2010). PCR was performed on the isolated DNA samples. Primers were designed using the domestic cat genome as a guide for exon 1, 5'-CACATCCTCTTGCCAGAACC-3' for ASIP exon 1 forward and 5'-GGGAGCACGTTTGACATCTT-3' for ASIP exon 1 reverse. For the remaining exons, we used primers from Eizirik et al 2003. Exon 2 forward (AgoEx3-F1) was 5'-TCCACTCCTCCCCACTTTACTG-3' and reverse (AgoEx3-R1) 5'-

CCCTTAGCTCTCTGGGCTTC-3'. The master mix for the reactions was composed of sterile water, 10X PCR Buffer Sigma with $MgCl_2$, 0.8 mM DNTPs, 0.4 mM of both the forward and reverse primers, and 0.2 units Red Taq (1 U/uL). The 18.5 uL of master mix was then added to 1.5 uL of each DNA sample for 20 uL reactions. The samples were then placed in a thermal cycler. A touchdown PCR profile was used: 1 minute denaturation at 94ºC, then 41 cycles of decreasing annealing temperature (2 cycles at 60ºC, 2 cycles at 58ºC, 2 cycles at 56ºC, 2 cycles at 54ºC, 2 cycles at 52ºC and finally 30 cycles at 50ºC), each cycle was preceded by 94ºC for 5 seconds and ended with 72ºC for 1 minute. The samples were then stored at 4ºC. After the PCR was complete, the samples were run on a 1% Agarose gel, and visualized using Ethidium Bromide (approximately 0.1% mg/uL) to allow for visualization of DNA under ultra violet light. All work was performed in the Equine Molecular Genetics & Cytogenetics Laboratory (Bhanu Chowdhary, Program Leader).

Sequencing

The resulting PCR was cleaned using the EXOSAP-IT PCR Clean-up Protocol, which digests unincorporated dNTPs and primers, 2 uL of ExoSAP-IT was added to every 5 uL of sample. The samples were incubated at 37ºC for 15 minutes and then 80ºC for 15 minutes to inactivate the ExoSAP-IT. We ran the standard Big Dye terminator reactions. Two uL of the cleaned up product was combined with 2 uL Big Dye v 1.1, and 1uL of the 2 ng/uL primers. This was then placed in the thermal cycler with the following program:

96ºC for 1 minute, 96ºC for 10 seconds for 25 cycles, 50ºC for 5 seconds, 60ºC for 4 minutes and then stored at 4ºC.

We removed the excess dNTPs using the Sephadex column method. The G50 Sephadex was loaded into the wells of a microtitre plate and then rehydrated with 300 uL double distilled water. The plate was spun at 2200 rpm for 5 minutes to pack the column. Then the Big Dye reactions were loaded on to the columns, along with 5 uL of water. The plate was then spun at 2200 rpm for 10 minutes. The cleaned up samples were lyophilized in a spin-vac, and rehydrated with 10 uL formamide and sequenced on the Applied Bio Systems 3730 DNA analyzer in the Chowdhary lab. Sequences were trimmed of poor quality bases and aligned using Sequencher 4.7 (Gene Codes) software.

We used the ASIP sequences from humans (NM-001672) and domestic cats (NM-001009190) to determine the intron/exon boundaries. We also pulled sequences from Genbank to allow for comparison between the felids we sequenced and several families of primates that diverged at a similar time. We pulled sequences for the Moor Macaque (*Macace maura),* the Celeces Crested Macaque *(Macaca nigra*), the Rhesus Macaque (*Macaca mulatta),* the Olive Baboon *(Papio Anubis*), the Blue Monkey *(Cercopithecus mitis),* the Silvery lutung *(Trachypithecus cristatus*), from the primate family Cercopithecidae, and the Human *(Homo sapiens)*, the Gorilla *(Gorilla gorilla),* and the Sumatran Orangutan *(Pongo abelii)* from the Hominidae primate family (See the results for accession numbers).

Sequences were aligned and analyzed using Clustal in MEGA 5. We generated a Maximum Likelihood Tree with the General Time Reversible Model, 500 bootstrap replicates and Nearest-Neighbor-Interchange Heuristic method. MEGA also generated a matrix using distance estimation with a number of different method, including transitions + transversions substitutions and uniform rates. We estimated diversity indexes of both nucleotide and haplotype diversities in DnaSP.

CHAPTER III

RESULTS

We extracted thirty-one samples following the PureGene blood extraction kit protocol. The isolation of DNA was confirmed using NanoDrop. The average quantity was 52.3 ng/uL. Each sample was also run on an agarose gel to examine the quality of the DNA and PCR amplified (Figure 1).

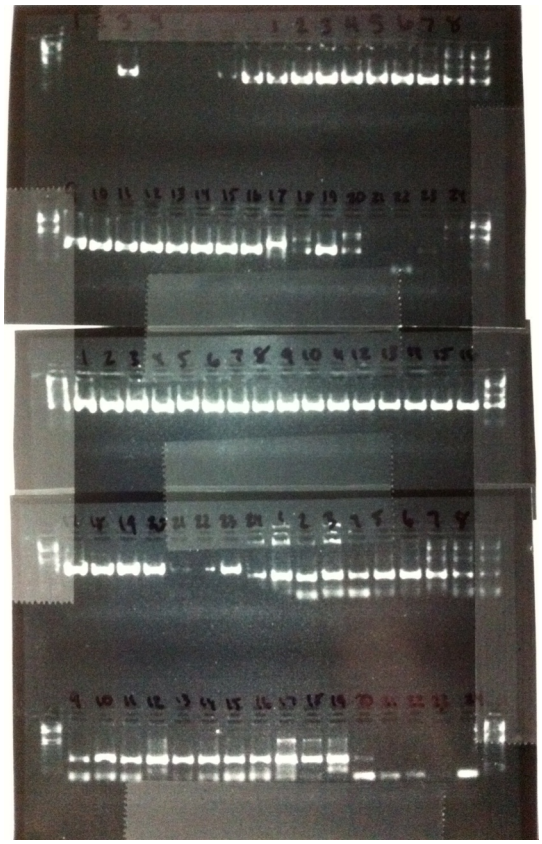

Figure 1. PCR results for ASIP exons 1-3

We sequenced ASIP exon 1 and 2 in thirty-one different samples, including twenty-four different tigers of several different colors; fourteen orange, four white, one snow white, four golden tabbies, and one tiger of unknown color. The remaining samples sequenced were from several different felids; two snow leopards, two cougars, two leopards and a jaguar. For exon 1 we obtained a 267 base pair long sequence after cutting out primers and for a 272 base pair sequence for exon 2. Exon 1 yielded a 160 base pair coding sequence leaving a 107 base pair section of the introns for analysis. For exon 2, the coding sequence was significantly shorter at 62 base pairs, leaving 210 base pairs from the introns for analysis. Exon 3 could not be sequenced, due to primer failure. This is likely due to a repeating section of DNA being situated in the middle of the primer. The primers were designed using the domestic cat genome, since the tiger has not been sequenced. The generated sequences from exon 3 were not very clear and looked as if the primer had annealed to multiple areas in the DNA. Therefore, we are redesigning primers that will work better with the tiger and re-sequencing ASIP exon 3.

Using MEGA, we first performed a phylogenic analysis of our data and sequences previously discovered. A Maximum likelihood tree using General Time Reversible Model + gamma was generated (Figure 2). The Maximum likelihood value generated for this tree was -945.70. Based on previous published phylogenetic trees, we determined the groups of primates that we wanted to examine and compare the felids to (Perelman et al. 2011). We chose to compare between felids and primates for several reasons. Primates are closely related to humans making them a common study animal; this has led to a great

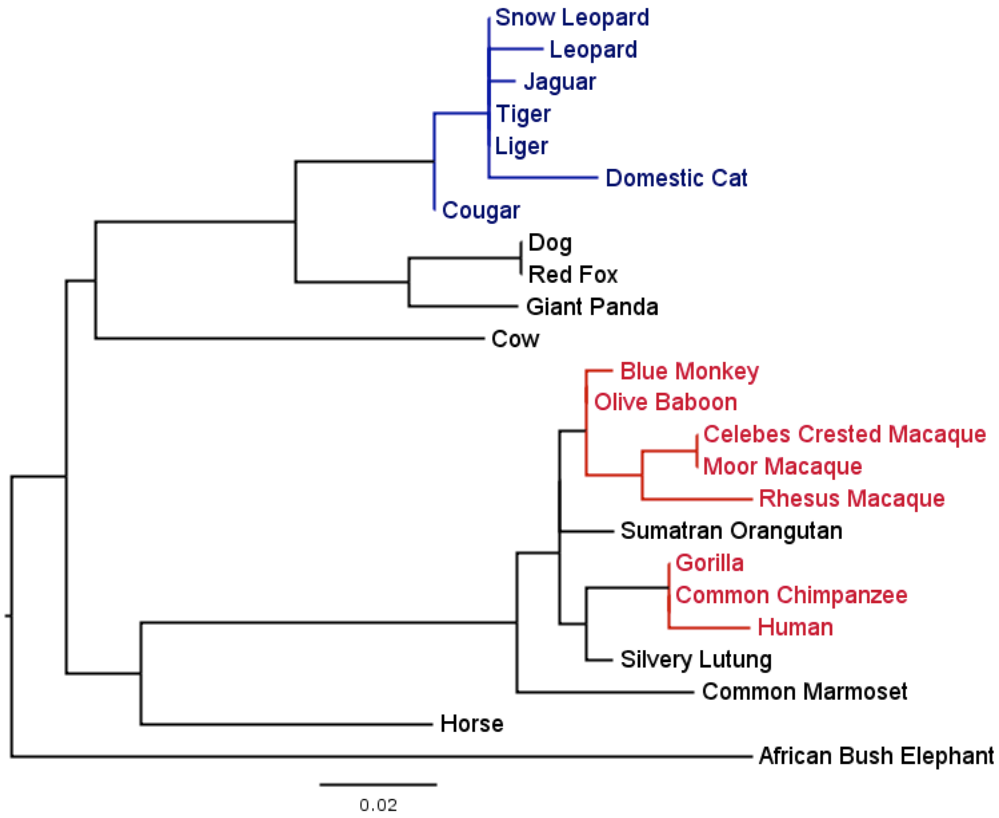

Figure 2. Maximum Likelihood Tree using general time reversal method with 500 bootstrap replicates. Maximum Likelihood value = -945.7

deal of information available about their genetics. We selected primates for our analysis that diverged at a similar time as the felids in our study. This makes them an excellent choice for comparing the variation we observed in felids. The primates we chose to compare are in the Cercopithecidae and the Hominidae primate groups; specifically *Macaca maura,* (AB299212)*, Macaca nigra* (AB299211), *Macaca mulatta* (AB299207), *Papio anubis* (NM-001164331)*, Cercopithecus mitis* (AB236877)*, Trachypithecus cristatus* (AB236880) from *Cercopithecidae,* and *Homo sapiens* (NM-001672), *Gorilla*

gorilla (AB236871), and *Pongo abelii* (XM-002830223) from Hominidae. All of these are Old World primates and diverged approximately 5.24 million years ago (Perelman et al. 2011). This is close to the 6.37 million years ago that the *Panthera* group diverged (Johnson et al, 2006). We created an alignment of the sequences for exon 1 and 2 and examined it for amino acid differences between felids and primates (Table 1). Table 1 shows the relationships between the different groups based on the alignment of their sequences. Comparing the felid sequences to the primate there was a total of 89 variable sites out of 228 base pairs. Based on our alignment we were able to determine that there were no differences in the amino acid sequences of ASIP exon 1 and 2 between the tiger, the liger, and the snow leopard (Figure 3A and B). We calculated nucleotide diversity between these groups, which equaled 0.0. The jaguar and the leopard each had one

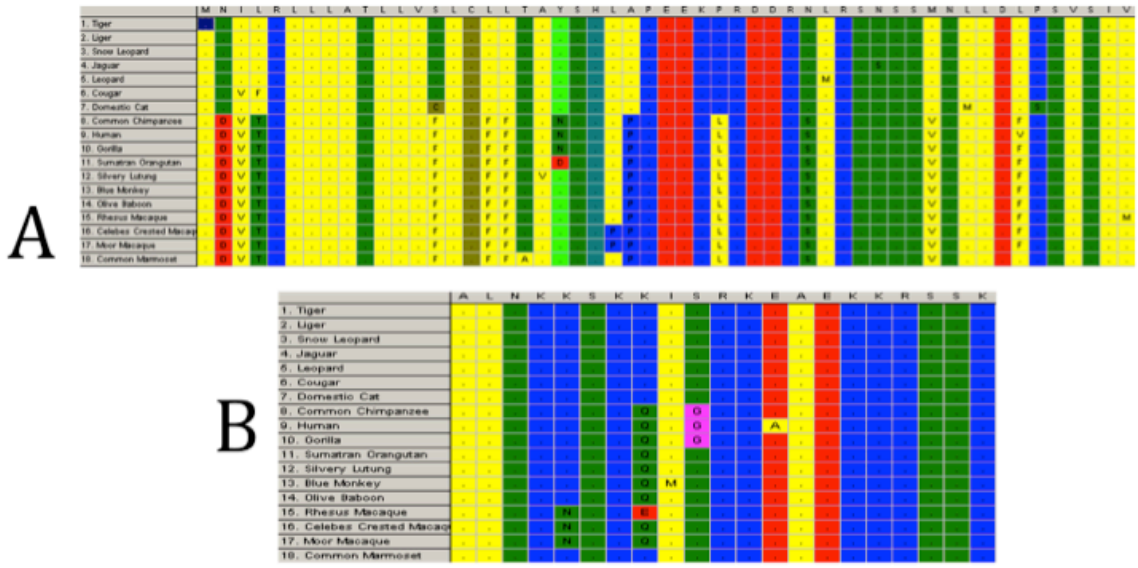

Figure 3. MEGA alignments for A) Exon 1 and B) Exon 2 of ASIP in felids and primates

different amino acid. The cougar and domestic cat, the most different from the other felids, had two and three differences respectively. Nucleotide diversity between all *Panthera we* sequenced was calculated to be 0.00558, with an average number of differences at 1.238. Interestingly, all of these differences occurred in exon 1. In exon 2 every felid sequence was the same. This is not true of the primates. When compared the Cercopithecidae and the Hominidae groups both averaged two amino acid differences (Table 1). The nucleotide diversity for Cercopithecidae was calculated to be 0.01982 and for Hominidae it was 0.00901. Cercopithecidae had an average number of differences was 4.4. In Hominidae the average number of differences was two. These amino acid changes were dispersed throughout the two exons.

Table 1. Comparison between different felids and primates

	Divergence Time	Nucleotide Subst. per Site	Amino Acid Subst. per Site	Number of Amino Acid Differences
Felidae	6.37			
Tiger vs. Snow Leopard	2.88	0	0	0
Tiger vs. Jaguar	3.72	0.005	0.014	1
Tiger vs. Cougar	10.8	0.009	0.028	2
Tiger vs. Leopard	3.72	0.009	0.014	1
Tiger vs. Domestic Cat	10.8	0.019	0.043	3
Primates	5.24			
Moor Macaque vs Celebes Crested	1.68	0	0	0
Moor Macaque vs Rhesus Macaque	4.13	0.028	0.043	3
Moor Macaque vs Olive Baboon	8.13	0.019	0.028	3
Moor Macaque vs Blue Monkey	11.5	0.023	0.043	2
Human vs. Chimpanzee	6.6	0.014	0.028	2
Human vs. Gorilla	8.3	0.014	0.028	2

We then examined the results from an alignment of just the sequences we obtained from the seven species that we sequenced. We then generated a figure to see the nucleotide differences between the different cats (Figure 4). Both the axes for this chart are the same, which allows for at a glance comparison between two animals.

Orange Tiger 1																													
Orange Tiger 2	0																												
Orange Tiger 3	0	0																											
Orange Tiger 4	0	0	0																										
Orange Tiger 5	0	0	0	0																									
Orange Tiger 6	0	0	0	0	0																								
Orange Tiger 7	0	0	0	0	0	0																							
Orange Tiger 8	0	0	0	0	0	0	0																						
Orange Tiger 9	0	0	0	0	0	0	0	0																					
Orange Tiger 10	0	0	0	0	0	0	0	0	0																				
Orange Tiger 11	0	0	0	0	0	0	0	0	0	0																			
Orange Tiger 12	0	0	0	0	0	0	0	0	0	0	0																		
Orange Tiger 13	0	0	0	0	0	0	0	0	0	0	0	0																	
White Tiger 1	0	0	0	0	0	0	0	0	0	0	0	0	0																
White Tiger 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0															
White Tiger 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0														
White Tiger 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0													
Snow White Tiger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0												
Golden Tabby Tiger 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0											
Golden Tabby Tiger 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0										
Golden Tabby Tiger 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0									
Golden Tabby Tiger 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
Unknow Color Tiger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0							
Liger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
Snow Leopard 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					
Snow Leopard 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
Jaguar	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
Leopard 1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3			
Leopard 2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	0		
Unknown	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	
Cougar 1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	4	4	2
Domestic Cat 1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	6	6	4

Figure 4. Number of Nucleotide differences in exon 1 and 2. Differences are highlighted in yellow.

CHAPTER IV

DISCUSSION

ASIP has three exons and a coding sequence of 408 base pairs total, which encodes a 136 amino acid protein. Exon 1 is 160 base pairs long followed by a 1652 long intron. Exon 2 is 61 base pairs followed by a 2329 base pair long intron. Lastly, exon 3 is 185 base pairs long. ASIP is a glycoprotein with a C-terminal that is rich in cysteine (McNulty et al. 2005). It is also highly conserved across mammalian species (Norris et al. 2012). ASIP is most commonly expressed in the skin, however in humans it has been found in humans in the liver, kidney, heart and adipose tissue (Voisey and van Daal 2002).

Cercopithecidae is a family of Old World monkeys, including baboons and macaques, that live throughout Africa and Asia in a variety of habitats ranging from savannah to rain forest. They are classified together because they have non-prehensile tails, unlike the apes, which have no tail, and the New World primates, which have prehensile tails (Delson 1975). Hominidae, another Old World primate family, are Great Apes – humans, gorillas, chimpanzees and orangutans – with no tails. The two families then diverged from each other approximately 31.56 million years ago (Perelman et al. 2011). These groups were chosen because they diverged similar to the felids that we are interested in. In the primates the most recent divergence time was 1.68 million years ago and the oldest was 11.5 million years (Table 1). This is similar to felids were the most recent was 2.88 million years ago and the oldest was 10.8 million years (Table 1).

The first analysis that we performed was centered on the phylogenic trees and tests that we performed by comparing the felids and primates. Figure 2 shows that the cougar and the domestic cat are the most different from the other felids, with the snow leopard, tiger and liger being identical. Among the primate families Cercopithecidae and Hominidae, the rhesus macaque and the human were the most different, whereas the celebes crested macaque and the moor macaque were identical, as were the gorilla and common chimpanzee. The variation among felids is very similar to the variation in primates. The average number of amino acid differences among the felids was 1.4. Among the primates the average number of amino acid differences was two. In felids and in Cercopithecidae there were nine variable sites, and in Hominidae there were three.

We compared in twenty-three different tigers, with four different coat color patterns, a 222 base pair segment of the coding sequence for ASIP exon 1 and 2. We determined that there are no differences between the different coat color phenotypes in the amino acid sequences of the ASIP gene for exon 1 and 2. This means there is likely no mutation in ASIP in exons 1 or 2 that is linked to the white coat color. This comparison generated one haplotype and no diversity at all in either the amino acid sequences or nucleotides.

However a mutation in ASIP could still be the cause of the white coat color. We did not generate good sequences for exon 3 and that could contain the relevant mutation. Therefore sequencing exon 3 will be the next step. Added to that we will be sequencing MC1R looking for significant mutations. We are also going to sequence the 5’ and 3’

untranslated regions (UTRs) of ASIP as well as MC1R. Sequencing the UTRs will allow us to determine if the cause of the white coat color is an issue with regulation of the proteins instead of expression of malformed proteins (Hughes 2006).

The ultimate goal of this project is to identify the gene that is responsible for the white coat color in tigers and determine if that gene is associated with the cause of defects that are common in white tigers. After sequencing MC1R and finishing sequencing ASIP exon 3, the next phase will be to genotype twelve microsatellites to determine the inbreeding coefficients for a variety of tigers with varying phenotypes. We will then see if inbreeding is correlated with coat color and with the defects associated with the white phenotype. This will allow us to determine if inbreeding is responsible for the physical defects seen so often in white tigers, or if they are instead the result of to the mutations that generated the allele itself.

In mice, ASIP has been linked with the lethal yellow mutation (Duhl et al. 1994). Heterozygotes are yellow in color, like the lethals, however they are viable. Heterozygosity for this mutation has also been linked to obesity, infertility, tumor susceptibility, and diabetes (Duhl et al. 1994). There is a similar mutation in Japanese quail (*Coturnix Japonica*), where the homozygous dominant is lethal (Nadaeu et al. 2008). The final goal of this project is to not only determine the gene associated with the white coat color in tigers, but to identify if that gene is associated with the defects (strabismus, spinal deformities, etc) that are commonly seen in the white coat color. In

Lethal White Foal Syndrome, completely white foals are born only to die after a few days because they possess a non-functioning intestinal tract. This is due to a mutation that affects the migration of cells from the neural crest (Thiruvenkadan et al. 2008). We believe that this could also be occurring in white tigers. The neural crest produces cells that contribute to a variety of different structures in the body, including the muscles, bones and nervous systems (Dupin et al. 2000). In Lethal White Foal Syndrome, the malformation of the neural crest prevents the migration of neural cells to the colon as well as pigment cells (Thiruvenkadan et al 2008). Therefore foals have non-functioning colons and no pigments. A similar development problem could be manifested in the white tiger; however, instead of the colon being affected it would alter the neural connections to muscles in the eyes, causing them to exhibit strabismus.

CHAPTER V

CONCLUSION

Our analysis of the Agouti Signaling Protein gene exon 1 and 2 showed that these are most likely not the cause of the white coat color in tigers. We plan to continue with sequencing MC1R to determine if it might be the culprit and we will design new primers and sequence ASIP exon 3. We are also planning on sequencing the 5' and 3'-untranslated regions around each gene. This will assist us in determining if a difference in expression of the pigments is the cause. We would be looking for a mutation in any regulatory sequences ahead of the gene. A mutation here would affect the binding of the transcription factor, potentially leading to an increase or decrease in these transcription of the gene.

This project is among the first steps into a very fascinating field. Many zoos, sanctuaries, and breeders are very interested in the genetics of their cats, and exhibit a great desire to maintain the tigers as best they can. Despite some controversy over the white coat color, everyone agrees we need to preserve tigers as best we can. By identifying the gene responsible for the coat color mutation, we can determine if the defects discussed in the introduction are due to inbreeding or if they are connected to the white phenotype allele itself, similar to Lethal White Foal Syndrome in horses. This information will could improve management and breeding strategies for captive tigers.

REFERENCES

Bultman, S., Michaud, E., Woychik, R., 1992. Molecular Characterization of the Mouse Agouti Locus. Cell. 71, 1195-1204.

Delson, E., 1975. Evolutionary History of the Cercopithecidae. In: Szalay, F.S. (Ed.), Approaches to Primate Paleobiology. Contributions to Primatology, vol. 5. Karger, Basel, pp. 167-217.

Dinerstein, E., Loucks, C., Wikramanayake, E., Ginsberg, J., Sanderson, E., Seidensticker, J., Forrest, J., Bryja, G., Heydlauff, A., Klenzendorf, S., 2007. The Fate of Wild Tigers. Bioscience. 57, 508–514.

Duhl, D., Vrieling, H., Miller, K., Wolff, G., Barsh, G., 1994. Neomorphic Agouti Mutations in Obese Yellow Mice. Nature. 8, 59-65.

Dupin, E., Creuzet, S., Le Douarin, N.M. 2006. The Contribution of the Neural Crest to the Vertebrate Body in: Saint-Jeannet, J. (eds.) Neural Crest Induction and Differentiation. Springer Science Business Media, New York, pp. 96-119.

Eizirik, E., Yuhki, N., Johnson, W., Menotti-Raymond, M., Hannah, S., O'Brien, S., 2003. Molecular Genetics and Evolution of Melanism in the Cat Family. Current Biology. 13, 448-453.

Fontanesi, L., Tazzoli, M., Beretti, F., Russo, V., 2006. Mutations in the Melanocortin 1 Receptor (MC1R) Gene are Associated with Coat Colours in the Domestic Rabbit (Oryctolagus Cuniculus). Animal Genetics. 37, 489–493.

Fontanesi, L., Forestier, L., Allain, D., Scotti, E., Beretti, F., Deretz-Picoulet, S., Pecchioli, E., Vernesi, C., Robinson, T., Malaney, J., Russo, V., Oulmouden A., 2009. Characterization of the Rabbit Agouti Signaling Protein (ASIP) Gene: Transcripts and Phylogenetic Analyses and Identification of the Causative Mutation of the Nonagouti Black Coat Color. Genomics. 95, 166-175

Fujita, P.A., Rhead, B., Zweig, A.S., Hinrichs, A.S., Karolchik, D., Cline, M.S., Goldman, M., Barber, G.P., Clawson, H., Coelho, A., Diekhans, M., Dreszer, T.R., Giardine, B.M., Harte, R.A., Hillman-Jackson, J., Hsu, F., Kirkup, V., Kuhn, R.M., Learned, K., Li, C.H., Meyer, L.R., Pohl, A., Raney, B.J., Rosenbloom, K.R., Smith, K.E., Haussler, D., Kent, W.J., 2010. The UCSC Genome Browser Database: Update 2011. Nucleic Acids Res. 39, D876-D882.

Hill, H.Z., Hill, G.J., Cieszka, K., 1997. Comparative Action Spectrum for Ultraviolet Light Killing of Mouse Melanocytes from Different Genetic Coat Color Backgrounds. Photochemistry & Photobiology. 65, 983–989.

Hughes T., 2006. Regulation of Gene Expression by Alternative Untranslated Regions. TRENDS in Genetic. 22 (3), 119-122.

Johnson, W., Eizirik, E., Pecon-Slattery, J., Murphy, W., Autunes, A., Teeling, E., O'Brien, S., 2006. The Late Miocene Radiation of Modern Felidea: a Genetic Assessment. Science. 311, 73-77.

Kerns, J., Newton, J., Berryere, T., Rubin, E., Cheng, J., Schmutz, S., Barsh, G., 2004. Characterization of the Dog *Agouti* Gene and a *Nonagouti* Mutation in German Shepherd Dogs. Mammalian Genome. 15, 798-808.

Lin, J.Y., Fisher, D.E., 2007. Melanocyte Biology and Skin Pigmentation. Nature 445: 843–850.

Menotti-Raymond, M., David, V.A., Eizirik, E., Roelke, M.E., Ghaffari, H., O'Brien, S.J., 2009. Mapping of the Domestic Cat "SILVER" Coat Color Locus Identifies a Unique Genomic Location for Silver in Mammals. Journal of Heredity. 100, S8-S13.

Mills, M., Patterson, L., 2009. Not Just Black and White: Pigment Pattern Development and Evolution in Vertebrates. Semin. Cell Dev. Biol. 20 (1), 72-81.

McNulty, J., Jackson, P., Thompson, D., Chai, B., Gantz, I., Barsh, G., Dawson, P., Millhauser, G., 2005. Structures of the Agouti Signaling Protein. Journal of Molecular Biology. 346, 1059-1070.

Nadeau, N., Minvielle, F., Ito, S., Inoue-Murayama, M., Gourichon, D., Follett, S., Burke, T., Mundy, N., 2008. Characterization of Japanese Quail Yellow as a Genomic Deletion Upstream of the Avian Homolog of the Mammalian ASIP (Agouti) Gene. Genetics. 178, 777-786.

Norris, B., Whan, V., 2012. A Gene Duplication Affecting Expression of the Ovine ASIP Gene is Responsible for White and Black Sheep. Genome Research. 18, 1282-1293.

Perelman, P., Johnson, W., Roos, C., Seuanez, H., Horvath, J., Moreira, M., Kessing, B., Pontius, J., Roelke, M., Rumpler, Y., Schneider, P., Silva, A., O'Brien, S., Pecon-Slattery, J., 2011. A Molecular Phylogeny of Living Primates. PLOS Genetics. 7 (3), 1-17.

Peter, D., Liu, Y., Sternini, C., de Giorgio, R., Brecha, N., Edwards, R.H., 1995. Differential Expression of Two Vesicular Monoamine Transporters. Journal of Neuroscience. 15, 6179–6188.

Peterschmitt, M., Grain, F., Arnaud, B., Deléage, G., Lambert, V., 2009. Mutation in the Melanocortin 1 Receptor is Associated with Amber Colour in the Norwegian Forest Cat. Animal Genetics. 40, 547–552.

Rieder, S., Taourit, S., Mariat, D., Langlois, B., Guerin, G., 2001. Mutations in the Agouti (ASIP), the Extension (MC1R), and the Brown (TYRP1) Loci and their Association to Coat Color Phenotypes in Horses. Mammalian Genome. 12, 450-455.

Robinson, R., 1969. The White Tigers of Rewa and Gene Homology in the Felidae, Genetica. 40, 198-200.

Robinson, R., 1990. Homologous Genetic Variation in the Felidae. Genetica. 46, 1–31.

Rozen, S. and Skaletsky, H.J., 2000. Primer3 on the WWW for General Users and for Biologist Programmers in: Krawetz, S. and Misener, S. (eds.) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365-386.

Roychoudhury, A.K. and Sankhala, K.S., 1979. Inbreeding in White Tigers. Proc. Indian Acad. Sci. 88B (5), 311-323.

Thiruvenkadan, A., Kandasamy, N., Panneerselvam, S., 2008. Coat Color Inheritance in Horses. Livestock Science. 117, 109-129.

Thornton, I., Yeung, K., Sankhala, K., 1967. The Genetics of the White Tigers of Rewa. Journal of Zoology, London. 152, 127-135.

Voisey, J. and van Daal, A., 2002. Agouti: From Mouse to Man, from Skin to Fat. Pigment Cell Res. 15, 10-18.

CONTACT INFORMATION

Name:	Emilee Ann Larkin
Professional Address:	c/o Dr. Jan Janecka Department of Veterinary Medicine and Biomedical Sciences MS 4458 Texas A&M University College Station, TX 77843
Email Address:	emlarkin03@gmail.com
Education:	B.A., Biomedical Sciences, Texas A&M University, May 2012 Honors Undergraduate Research Fellow